ISSN 0825-4591

PROCEEDINGS

ENVIRONMENTAL RESEARCH:

1991 TECHNOLOGY TRANFER CONFERENCE

November 25 & 26, 1991

Four Seasons
Inn on the Park

Toronto, Ontario

VOLUME II

FEATURE PRESENTATIONS

EMISSIONS AND EFFLUENTS

ENVIRONMENTAL TECHNOLOGIES PROGRAM

PREVENTION, TREATMENT AND REMEDIATION

TECHNOLOGIES/BIOTECHNOLOGY

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Environment Ontario

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DEVELOPMENT OF AN ENZYME IMMUNOASSAY FOR THE RAPID DETECTION AND QUANTIFICATION OF GLYPHOSATE

G.M. Charbonneau, T.L. Collier, W.G. Craig, and C.J. Morin, Paracel Laboratories Ltd., Ottawa, ON, K1G 4K6, and C.D. Hall, Laboratory Services Branch, Ontario Ministry of the Environment, Rexdale, ON, M9W 5L1.

INTRODUCTION

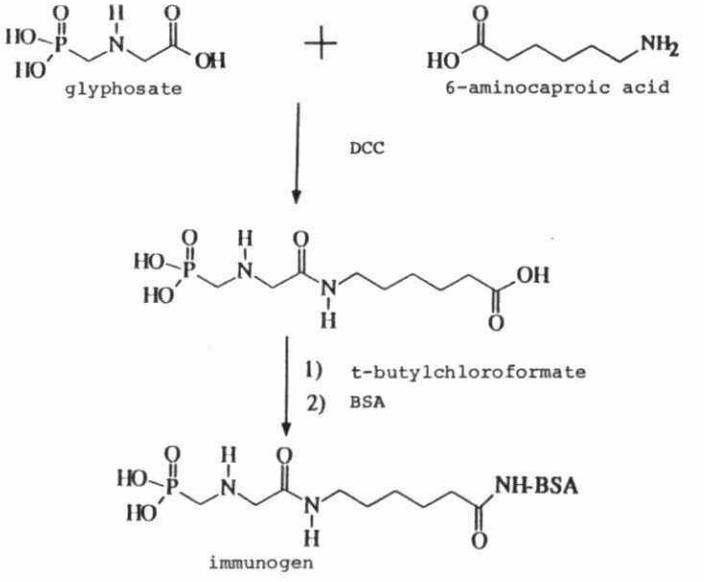
Glyphosate (N-phosphonomethylglycine) is a broad spectrum, non-selective, post-emergence herbicide; it is effective in the control of a wide range of weeds and at the same time is relatively non-toxic to mammals. The LD_{50} based on oral feeding of male rats was reported to be 4320 mg/kg (Watts, 1980). The currently accepted LD_{50} value for mammals is 1568 mg/kg (Miles *et al.*, 1986). Properties which make this compound effective include high water solubility, rapid absorption and translocation by plants, and low degrees of *in vivo* metabolism and degradation. It is a unique compound in that it acts by disruption of phenolic metabolism (Hoagland and Duke, 1981).

Because of the significance of glyphosate, sensitive, accurate and widely applicable analytical methods for glyphosate are essential. Special complexities arise in the quantitation of this compound because it is soluble in water and insoluble in organic solvents. Currently, the detection of glyphosate is most commonly performed by biphasic aqueous-organic extraction followed by cleanup of the supernatant aqueous extract utilizing, first, iron-loaded Chelex 100 resin, and then, AG-1-X8 anion-exchange resin columns. Liquid chromatography coupled to a postcolumn reactor specific for primary amines, or compounds that can be converted to primary amines, is then used for the quantitation of the extract (Cowell *et al.*, 1986). This analytical technique is long , costly, and has a detection limit near 0.05 ppm and recoveries around 80%. The use of an enzyme immunoassay would be of great help in *i*) shortening the time required for the analysis, *ii*) allowing the simultaneous analysis of many samples at the same time, and *iii*) lowering the detection limit. This research project is directed to the development of an enzyme immunoassay against glyphosate using polyclonal antibodies that could possibly, later be replaced by monoclonal antibodies. It is divided into three major stages which include: *i*) the preparation of the immunogen, *ii*) the production and selection of polyclonal antibodies and, *iii*) the preparation of a tracer and the development of a competitive enzyme immunoassay. Currently, part i) and some of part ii) have been achieved.

MATERIALS, METHODS AND RESULTS

1. SYNTHESIS OF THE BOVINE SERUM ALBUMIN-GLYPHOSATE CONJUGATE

As glyphosate is too small a molecule to be immunogenic, the first step in the production of an immunoassay for glyphosate is the synthesis of an immunogen consisting of the hapten, glyphosate, covalently linked to a large carrier molecule such as bovine serum albumin (BSA). The antibodies raised against such an immunogen tend to have highest specificity against that part of the hapten molecule that is furthest removed form the carrier. This consideration caused us to link glyphosate via the carboxylic acid function of the glycine moiety which will allow as much as possible of the phosphonate group to be exposed during antibody production. Molecular modelling calculations that predict the degree of steric hindrance of the hapten and the optimum electronic distributions for antibody formation indicated that the hapten should be attached to BSA via a five to six carbon chain rather than directly.



This was accomplished using 6-aminocaproic acid in the presence of 1,3-dicyclohexylcarbodiimide (DCC) to form an amide. The amide was purified by preparative thin layer chromatography and methanol recrystallization and its identity confirmed by nuclear magnetic resonance and mass spectrometry. The glyphosate-caproic conjugate was attached to BSA by a mixed anhydride method using t-butylchloroformate. The resulting immunogenic conjugate was purified by dialysis against distilled water and subsequent lyophilization.

2. POLYCLONAL ANTIBODY PRODUCTION AND SELECTION

A set of six rabbits was used for the immunization. Blood samples were taken through the ear at DAY 0 in order to obtain pre-immune serum from each of the rabbits. They were then injected with 0.5 mL of a mixture 1:1 of the conjugate in PBS and complete Freund adjuvant (approx. 1 mg/mL). Four (4) injections of 0.5 mL each were made on each animal at the same time, two injections sub-cutaneously and two injections intra-muscularly. On DAY 21, 42, and 63, the rabbits were re-injected with the same amount of conjugate, but this time, mixed 1:1 with incomplete Freund adjuvant. On DAY 73, each rabbit was sampled through the ear and tested for the presence of anti-BSA antibodies using simple immunodiffusion. Since no reaction was observed, the six rabbits were re-injected twice following the same protocol in an attempt to raise the titer. They were then re-tested by immunodiffusion against BSA and also the BSA-glyphosate conjugate. Again, there was no reaction observed between the sera and the BSA alone. However, for two rabbits, precipitation bands were observed between the sera (rabbits number 3 and 6) and the BSA-glyphosate conjugate. These two rabbits were then bled by intra-cardiac puncture. Their sera is now being tested for specificity against glyphosate using a radioimmunoassay (RIA).

The RIA testing of the rabbit sera is as follow. Polyvinyl chloride microtitration plates are coated with 100 µL of a 1,000-fold dilution in PBS (20 mM) of swine anti-rabbit immunoglobulins (Dakopatts, Denmark) and incubated overnight at room temperature (RT). Uncoated plastic is then saturated by adding 100 µL of PBS-BSA (6%) to the wells. After an incubation of 3 h at RT, the plates are washed three times with PBS and 100 µL of serial dilutions, in PBS, of the rabbit antisera are placed in wells. The plates are left at RT for 3 h. They are then washed four times with PBS-Tween 20 (0.05%) (PBST) and filled with 100 µL of ^{14}C radiolabelled glyphosate (approx. 50,000 cpm) in PBST. After 3 h of incubation at RT, the plates are washed six times with PBST and each well is cut and placed in a liquid scintillation vial containing 12 mL of Beckman's Ready Protein^{+} cocktail. Radioactivity present in each well is then estimated using a Packard 1900CA Liquid Scintillation Analyzer. Antibody titer is

defined as the reciprocal of the amount of antisera (mL) required to give 50% binding of radioactive glyphosate.

Results were not yet available from the RIA testing when the Proceedings manuscripts were required.

DISCUSSION

The power of immunochemical technology to solve problems in the pesticide field is becoming widely appreciated. Immunoassays are physical assays which offer many advantages, including simplicity, sensitivity and specificity. Immunoassays do not rely on volatility (GC), thermal stability, or the presence of chromophores (UV, fluorescence) or heteroatoms (ECD, NPD) for detection. The development of competitive enzyme immunoassays is one of the most promising approaches to the detection and characterization of both xenobiotics and biological entities. Not only does enzyme immunoassay development allow the lowering of detection limits for environmental pollutants, but it also allows spending less time doing the routine runs and permits its application in less fully equipped laboratories. Since immunoassays usually work well in human body fluids, the technology is applicable to evaluation of worker exposure as well. Thus, immunochemistry is likely to complement existing technologies in providing, at reduced cost, the type of data currently generated in pesticide trace analysis.

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